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Dr. Baumeister

Editor-in-Chief

Biochemical and Biophysical Research Communications

Dear Dr. Baumeister,

We would like to submit our manuscript titled "**Development of complete human IgG monoclonal antibody to transferrin receptor 1 for adult-T cell leukemia/lymphoma**" by **Shunsuke Shimosaki, Shingo Nakahata, Tomonaga Ichikawa, Akira Kitanaka, Takuro Kameda, Tomonori Hidaka, Yoko Kubuki, Gene Kurosawa, Lilin Zhang, Yukio Sudo, Kazuya Shimoda, Kazuhiro Morishita** for publication as a regular paper in *Biochemical and Biophysical Research Communications*.

Adult T-cell leukemia/lymphoma (ATLL) is a refractory T-cell malignancy associated with HTLV-1 infection. Despite recent progress in ATLL therapy including anti-CCR4 antibody (mogamulizumab), the long-term prognosis of ATLL patients remains unsatisfied due to the high rate of relapse and disease resistance to chemotherapy, and therefore, to develop novel molecular targets for treatment of ATLL, we have identified several candidate cell surface molecules including transferrin receptor 1 (TFR1), which was highly expressed on the surface of acute-type ATLL cells by DNA microarray analysis (*1*). Therefore, in this manuscript, we developed complete human IgG monoclonal antibody to human TFR1, termed JST-TFR09, which is highly sensitive to TFR1 on the surface of ATLL cells. Treatment of ATLL cells or other leukemia cells with JST-TFR09 significantly suppressed cell growth and survival much more effectively than other anti-TFR1 antibodies, such as the 42/6 antibody that has been shown as the most potent cytotoxic effects against leukemia cells with phase Ia clinical trial (2). Moreover, we show that JST-TFR09 elicits antitumor effects with apoptosis not only by blocking iron uptake but also by inducing ADCC against ATLL cells. Furthermore, treatment of ATLL cell xenograft mice with JST-TFR09 completely inhibited the ATLL tumor growth, whereas mogamulizumab partially suppressed it. Therefore, JST-TFR09 might become a promising therapeutic agent that prevents the progression of ATLL.

We think that this manuscript is of importance as a potential treatment for ATLL and towards improved its therapy. This paper also provides an important knowledge to the field of cancer biology. All co-authors have approved the manuscript and agreed to submit it to Biochemical and Biophysical Research Communications, and there is no financial interest or conflicts to report. We certify that this manuscript is not under review by any other publication.

Thank you in advance for considering our paper and we are looking forward to receiving your reply.

Sincerely yours,

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References

1. H. Sasaki, I. Nishikata, T. Shiraga, et al., Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. Blood 105 (2005) 1204-1213.
2. Brooks D, Taylor C, Dos Santos B, Linden H, Houghton A, Hecht TT, Kornfeld S, Taetle R. Clin Cancer Res. Phase Ia trial of murine immunoglobulin A antitransferrin receptor antibody 42/6. 1 (1995) 1259-65.

Highlights

- ATLL cells express high levels of TFR1 and shows enhanced iron dependency.
- Newly isolated anti-TFR1 mAb, JST-TFR09, is highly reactive to TFR1 on ATLL cells.
- JST-TFR09 can act via blockade of cellular iron uptake but also ADCC to ATLL cells.
- JST-TFR09 efficiently suppresses ATLL cell growth in vitro and in vivo mouse model.
- JST-TFR09 may be a better therapeutics antibody than anti-CCR4 antibody for ATLL.

Development of a complete human IgG monoclonal antibody to transferrin receptor 1 targeted for adult T-cell leukemia/lymphoma

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Key words

Anti-transferrin receptor 1 antibody; Adult T-cell leukemia/lymphoma; Phage display; Direct cytotoxicity; ADCC

Abstract

Iron is an essential nutrient for normal cell growth, and reprogramming of iron metabolism is essential to tumor cell survival and progression. HTLV-1-associated adult T-cell leukemia/lymphoma (ATLL) has no effective therapy and high levels of cell surface transferrin receptor 1 (TFR1) expression have been reported in ATLL by us and other groups. In this study, to develop a novel molecular-targeted therapy against TFR1 to modulate iron metabolism, we initially determined the expression pattern of several iron-related genes along with TFR1 and found that ATLL cells presented characteristic of an iron-deficiency state such as high expression of iron-regulatory protein 2 (IRP2) and low expression of its E3 ubiquitin-ligase, FBXL5. Therefore, we developed human IgG monoclonal antibodies to human TFR1 using a phage display method (ICOS method) to block the incorporation of the transferrin (TF)-iron complex into ATLL cells for inhibiting cell growth. One of the mAbs, JST-TFR09, presented its greater affinity to TFR1 on ATLL cells in flow cytometry (FCM) analysis than those of commercially available anti-TFR1 antibodies and identified high expression of TFR1 in most of the acute-type ATLL cells. Moreover, JST-TFR09 could interfere with binding between TFR1 and TF, which resulted in effective blockade of TFR1 internalization and induction of cell apoptosis by the treatment of ATLL cells with JST-TFR09. JST-TFR09 showed dual activities through direct cell cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), and the treatment of JST-TFR09 significantly suppressed cell growth of ATLL cells with induction of apoptosis in *in vitro* and *in vivo* experiments.

Thus, JST-TFR09 described here may become a promising therapeutic antibody for the treatment of ATLL.

1. Introduction

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive malignancy caused by human T-cell leukemia virus type 1 (HTLV-1). HTLV-1 is endemic to certain regions, including Japan, Africa, the Caribbean and South America, and an estimated 10-20 million people worldwide are currently infected with HTLV-1. HTLV-1 infects primarily $CD4^+$ T-lymphocytes through direct cell-to-cell contact, mainly via breast-feeding. ATLL develops in 3-5% of HTLV-1 carriers after a long latency period of 20-60 years during which polyclonal expansion of HTLV-1-infected lymphocytes predisposes them to transformation [1]. ATLL is classified into four clinical types: acute, chronic, lymphoma, and smoldering. Patients with aggressive forms (acute and lymphoma) have a very poor prognosis because of intrinsic chemoresistance and severe immunosuppression. Although new therapeutic options such as allogeneic hemopoietic stem-cell transplantation (allo-HSCT), interferon-α (IFN-α) plus zidovudine (AZT), and the anti-CCR4 monoclonal antibody (mAb) are gradually improving the curability of aggressive ATLL, treatment still remains challenging [2]. Patients with indolent ATLL (chronic or smoldering) have a better prognosis than the aggressive type of ATLL. However, recently, a poor long-term outcome has been demonstrated when patients are managed with a watchful waiting policy [3]. Thus, there is an urgent need to develop new strategies to increase the effectiveness of treatments for this disease.

To search for novel cell surface target molecules for ATLL, we previously studied gene expression profiles between acute-type ATLL leukemia cells and control $CD4^+$

T-lymphocytes by DNA microarray analysis and identified cell adhesion molecule 1 (TSLC1/CADM1), interleukin 2 receptor subunit α (IL2RA), tumor necrosis factor receptor superfamily member 6 (TNFRSF6), fibroblast growth factor receptor 1 (FGFR1), and transferrin receptor 1 (TFRC/TFR1/CD71) as significantly differentially expressed genes in ATLL [4]. TFR1 has been suggested to be one of the promising ATLL cell surface target molecules [5]. In this manuscript, we present work on the development of an anti-TFR1 antibody for the treatment of ATLL. TFR1 plays an important role in the maintenance of iron homeostasis by regulating iron uptake through binding and internalizing the iron-transferrin (TF) complex. Because iron is an essential nutrient required to maintain cell growth, elevation of iron metabolism is often associated with cancer cell growth and survival. Indeed, TFR1 expression is frequently up-regulated in various cancer cells including ATLL, which is often associated with poor prognosis [5-9]. On the other hand, in normal cells, TFR1 is expressed at low levels but is expressed at greater levels on some particular cell types, such as erythroid lineage cells [10]. To date, several kinds of anti-TFR1 antibodies have been developed for cancer diagnosis and targeted therapy, and some of them have been tested in clinical trials. A mouse IgA mAb against TFR (42/6) has been evaluated in a phase I clinical trial, and 3 cases among the 27 patients with advanced refractory cancers with hematologic malignancies showed evidence of mixed tumor responses [11]. Recent studies have shown that a mouse mAb against TFR1 (A24) can inhibit proliferation of ATLL cells through induction of apoptosis [5] and that human single-chain antibodies to TFR1 effectively antagonize the

growth of hematopoietic tumor cell lines of various lineages [12]. However, there are no available complete human IgG mAbs directed against TFR1 that can be used in the clinic.

To develop complete human IgG mAbs against human TFR1 for use in novel therapies for ATLL, we screened human antibody phage display libraries using a method called isolation of antigen-antibody complexes through organic solvent (ICOS) and isolated several anti-TFR1 mAbs [13,14]. We previously reported that one of the mAbs showed cytotoxic activity against oral squamous cell carcinoma (OSCC) cells [15]. In an effort to obtain additional anti-TFR1 mAbs that are more effective than other mAbs, such as anti-CCR4 and anti-TFR1 A24, in treating ATLL, we further screened the phage antibody library and succeeded in isolating different anti-TFR1 mAbs. Here, we used one of the mAbs, JST-TFR09, which showed the strongest cytotoxic activity against ATLL cells, to characterize its therapeutic potential for ATLL in vitro and in vivo. The results showed that JST-TFR09 presented direct cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) activity to ATLL cells and significantly better antitumor effects in vitro and in vivo compared with that of the anti-CCR4 antibody, suggesting that the anti-TFR1 antibody will become a promising therapeutic agent for ATLL.

2. Materials and methods

2.1. Patient samples

Blood samples were collected from ATLL patients at the time of hospital admission

before chemotherapy. The diagnosis of ATLL was based on clinical features, hematological characteristics and the presence of anti-HTLV-1 antibodies in the sera. Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers and ATLL patients were purified by gradient centrifugation using Histopaque St. Louis, MO). After the purification of $CD4^+$ cells form PBMCs using anti-CD4 magnetic beads from Miltenyi Biotec (Bergich Gladbach, Germany), ATLL cells were collected using the biotin-conjugated CADM1 antibody [16] together with anti-biotin magnetic beads (Miltenyi Biotec, Auburn, CA) after purification of T-cells using the T Cell Isolation Kit (Miltenyi Biotec). ATLL cells were maintained in AIM-V medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 20% FBS, 10 μM 2-mercaptoethanol (Thermo Fisher Scientific) and 0.75 μg/mL recombinant human IL2 (Pepro tech) in a humidified atmosphere of 5% CO_2 at 37 °C. Informed consent was obtained from all patients. This study was approved by the Institutional Review Board of the Faculty of Medicine, University of Miyazaki.

(The remaining materials and methods are presented in the supplementary information.)

3. Results

3.1. TFR1 is overexpressed in ATLL cells

Because analysis of our microarray data revealed up-regulation of TFR1

expression in leukemic cells from ATLL patients (Fig. S1), we performed quantitative RT-PCR analysis for TFR1 using 9 HTLV-1-positive ATLL-related cell lines, 2 HTLV-1-negative T-cell acute lymphoblastic leukemia (T-ALL) cell lines, primary leukemic cells from 9 acute-type ATLL patients, and $CD4^+$ T-lymphocytes from 4 healthy volunteers as controls (Fig. 1A). The majority of the ATLL-related cell lines, T-ALL cell lines, and primary ATLL cell samples showed more than a 5-fold increase in TFR1 mRNA compared to that of controls. In an immunoblot analysis, we observed that the TFR1 protein was abundantly expressed in all of the ATLL-related cell lines and primary ATLL cell samples, while $CD4^+$ T-lymphocytes from healthy volunteers expressed the TFR1 protein at a very low level (Fig. 1B). In addition, we used the human anti-human TFR1 mAb (JST-TFR09), which was the antibody isolated from the antibody phage display library screening that had the highest binding affinity and cytotoxic activity for the development of a better therapeutic antibody than anti-CCR4 or a mouse anti-TFR1 A24 mAb, to perform flow cytometry (FCM) analysis. The results showed that the vast majority of ATLL cells expressed high levels of TFR1 on their surface (Tables S1 and S2 and Fig. 1C). We also found that JST-TFR09 had much greater sensitivity for ATLL cells than the commercial antibodies (Fig. S2).

Because iron metabolism is reprogrammed in cancer cells for enhanced cellular proliferation [17], we next examined whether the genes involved in iron metabolism are differentially expressed in ATLL cells. As shown in Figs. S3 and S4, mRNA levels of the E3 ubiquitin ligase subunit, FBXL5, and the iron exporter, FPN, were significantly decreased in most of the ATLL-related cell lines and primary ATLL cells, whereas levels

of the heavy subunit of ferritin (FTH1) and IRP2 mRNA were significantly up-regulated compared to that of the $CD4^+$ T-lymphocytes from healthy volunteers. On the other hand, the ferritin light chain (FTL), IRP1, and DMT1 mRNA levels were not significantly different between ATLL cells and normal $CD4^+$ T-cells. Moreover, we found that the levels of FBXL5 protein were consistently decreased in all of the ATLL-related cell lines and primary ATLL cell samples, and, conversely, levels of the IRP2 protein were highly up-regulated in these cells (Fig. 1B), suggesting that the increased steady-state levels of IRP2 caused by down-regulation of FBXL5 expression could contribute to sustained up-regulation of TFR1 in ATLL cells, resulting in an increase in the labile iron pool that may support cell growth.

Because epigenetic alteration is one of the important mechanisms leading to deregulation of gene expression in ATLL, we next determined whether down-regulation of FBXL5 is associated with epigenetic modification of the FBXL5 locus. As shown in Fig. S5, treatment with the histone deacetylase inhibitor trichostatin A (TSA) partially recovered FBXL5 expression in both the HTLV-1-infected and ATLL cell lines, while the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza) minimally activated its expression. These results suggest that histone deacetylation is associated with the repression of FBXL5 expression in ATLL.

3.2. The anti-TFR1 antibody can kill ATLL cells in vitro

To determine the therapeutic potential of JST-TFR09, we first examined its direct cytotoxic activity in ATLL-related cell lines, leukemic cells from ATLL patients, and

$CD4^+$ T-lymphocytes and erythroblasts from healthy volunteers using an a tetrazolium (MTT) colorimetric assay. As expected, no significant cytotoxic effect was observed in resting $CD4^+$ T-lymphocytes after treatment with JST-TFR09 (Fig. 2A). On the other hand, JST-TFR09 treatment of normal erythroblasts and PHA-activated $CD4^+$ T-cells, both of which showed high-level expression of TFR1, caused a dose-dependent decrease in cell viability with an IC50 value of 125 ng/mL and 107 ng/mL, respectively (Fig. 2A, Fig. S6 and Table S1). Notably, JST-TFR09 strongly reduced survival of all of the HTLV-1-infected and ATLL cell lines with an IC50 value of approximately 10-50 ng/mL (Fig. 2B and Table S2), which was accompanied by an increased rate of apoptosis (Fig. S7). Importantly, primary leukemic cells from ATLL patients were also highly sensitive to the cytotoxic effect of JST-TFR09 with an IC50 value of approximately 20-150 ng/mL (Fig. 2C and Table S1). JST-TFR09 was also highly effective for other types of leukemia, such as acute myeloid leukemia (Table S3).

To clarify whether the cytotoxic effect of JST-TFR09 against ATLL cells is mediated by the blockade of uptake of the iron-TF complex, we first performed an enzyme-linked immunosorbent assay (ELISA)-based competitive binding assay wherein different concentrations of JST-TFR09 or unlabeled holo-TF were added to the reaction plate pre-coated with soluble TFR1 and then horseradish peroxidase (HRP)-conjugated holo-TF (Fig. 3A and Table S4). The binding affinity between JST-TFR09 and TFR1 was significantly higher than that between holo-TF and TFR1 (IC50 = 0.04 versus 6.37 μg/mL). In the next experiment, to confirm the high affinity of the JST-TFR09 antibody for TFR1 on the cell surface of leukemic cells, K562 erythroleukemia cells were

incubated with fluorescein-labeled holo-TF (FL-holo-TF) in the presence of various concentrations of JST-TFR09 or unlabeled holo-TF, and the incorporation of FL-holo-TF into the cells was determined by FCM analysis. The FL-holo-TF accumulation in K562 cells preincubated with JST-TFR09 was lower than that in cells preincubated with holo-TF (Fig. 3B and Table S5), suggesting that the binding affinity between JST-TFR09 and TFR1 is much stronger than that between holo-TF and TFR1. We also determined the kinetics of TF internalization in MT-2 cells preincubated with or without JST-TFR09 (Fig. 3C). While the incorporation of holo-TF by MT-2 cells gradually increased during the 10-minute incubation period, only a subtle increase in holo-TF incorporation was detected in the cells treated with JST-TFR09 over the same time period. Based on these results, we suggest that treatment with the JST-TFR09 antibody efficiently inhibits iron-TF uptake, thereby triggering cell death.

To further define the cytotoxic properties of JST-TFR09, we next examined the ADCC activity of JST-TFR09 against ATLL-related cell lines in the presence of peripheral blood mononuclear cells (PBMCs) from healthy volunteers, since the antitumor activity of the anti-CCR4 antibody (mogamulizumab) against ATLL cells is primarily dependent on the ADCC [18]. The ADCC activity of JST-TFR09 was less than that of mogamulizumab against MT-2 cells; however, similar levels of ADCC activity between JST-TFR09 and mogamulizumab were detected in S1T cells, suggesting that JST-TFR09 can elicit its effects via two independent mechanisms, cytosolic iron depletion by disrupting TFR1 function and cytotoxic activity via ADCC.

3.3 The anti-TFR1 antibody can inhibit ATLL tumor growth in vivo

To examine the effects of JST-TFR09 on the growth of ATLL cells in vivo, we used an immunodeficient NOD/Shi-scid/IL-2Rγnull (NOG) mouse subcutaneously transplanted with the HTLV-1-infected cell line MT-2 and examined the antitumor activity of JST-TFR09 in comparison with that of the anti-CCR4 antibody mogamulizumab. JST-TFR09, mogamulizumab, or PBS as control were injected intravenously 10 days after MT-2 cell inoculation, and tumor growth was monitored every 3 days for a 3-week period. As shown in Fig. 4A, the JST-TFR09 mAb completely blocked tumor growth, whereas mogamulizumab had only marginal effects because the immunodeficient NOG mice largely lacks functional natural killer (NK) cells and monocytes. Moreover, JST-TFR09 mAb-treated mice showed markedly extended survival that was significantly different from that of the PBS-treated mice (Fig. 4B and Fig. S8). To further verify the effects of JST-TFR09, we injected JST-TFR09, mogamulizumab, or PBS into SCID mice, which have functional NK cells and monocytes, transplanted with the human cutaneous T-cell lymphoma (CTCL) cell line HH expressing both TFR1 and CCR4. No tumor growth was observed in JST-TFR09-injected mice, while the tumor volume increased slowly but steadily with time in mogamulizumab-injected mice (Fig. 4C). Collectively, these results suggest that JST-TFR09 produces a strong anti-tumor activity against ATLL cells and might become a promising therapeutic agent in ATLL.

4. Discussion

In this manuscript, we initially confirmed high expression of TFR1 along with the iron-storage protein ferritin and down-regulation of the iron exporter FPN in ATLL. Moreover, the IRP2 protein, an iron-dependent post-transcriptional regulator, was highly accumulated in ATLL cells, and the FBXL5 protein, an E3 ubiquitin ligase for IRP2, was almost completely degraded, suggesting that ATLL cells are under iron-deficient and/or hypoxic conditions. Since the reprogramming of iron metabolism is critical for tumor cell survival and growth, inhibition of cellular iron uptake by the anti-TFR1 antibody could be a promising strategy for prevention of cancer progression.

High expression levels of TFR1 as well as ferritin and low FPN expression levels have been reported in a variety of cancers such as breast, pancreatic, and hepatocellular cancers [19]. The increased levels of labile iron in the cytosol can catalyze the formation of reactive oxygen species, which can contribute to carcinogenesis either directly through genotoxic effects or indirectly via modification of signaling pathways including Wnt, HIF-1α, AP-1, NF-κB, and p53 [20]. It has been recently proposed that cancer cells exhibit an enhanced dependence on iron for growth and are more susceptible to iron deficiency than noncancer cells [19]. Our studies also revealed that the down-regulation of FBXL5 expression, at least in part, through histone deacetylation is associated with enhanced TFR1 expression via enhanced IRP2 degradation in ATLL cells. The large HECT-type ubiquitin ligase HERC2, which is essential for DNA damage repair pathways, was recently reported to regulate the basal turnover of FBXL5 via a ubiquitin-dependent degradation pathway [21]. Interestingly,

levels of HERC2 mRNA were also found to be elevated in leukemic cells from about half of the patients with acute ATLL (data not shown). Further studies are needed to clarify the involvement of HERC2 in FBXL5 regulation in ATLL.

For cancer therapy, TFR1 is an important target not only for drug delivery into cancer cells but also blockade of iron uptake by antagonizing TFR1 function, which leads to cancer cell death [22]. Therefore, a number of anti-TFR mAbs have been developed and tested for their inhibitory effects on cancer cells in in vitro and in vivo mouse models of several cancer types. Among them, the mouse IgA anti-human TFR mAb 42/6 has demonstrated the most potent cytotoxic effects against human malignancies. After in vitro studies using myeloid leukemia cells, a phase I clinical trial was conducted in 27 patients with advanced refractory cancer of different origins (epithelial, mesenchymal, and hematopoietic) [11,23]. Although 42/6 infusions resulted in suppression of malignant cell growth with minimal nonspecific effects, favorable therapeutic results were not achieved because 42/6 was rapidly cleared from the peripheral blood due to its mouse origin. Additionally, an anti-mouse IgA antibody was developed in the patient's blood. To determine whether the cytotoxic ability of 42/6 is comparable to that of JST-TFR09, we used three leukemia and lymphoma cell lines (K562, HL60, and SU-DHL-2) and included a mouse IgG1 anti-TFR1 A27.15 mAb, which inhibited the growth of IL6-independent human myeloma cells [24]. The IC50 value of JST-TRF09 ranged from 4.6 to 65 ng/mL; however, that of 42/6 and A27.15 ranged from 237 ng/mL to over 100 μg/mL and 341 ng/mL to over 100 μg/mL, respectively (Table 3), suggesting that the previously used antibodies for TFR1 were

significantly less sensitive than JST-TFR09.

JST-TFR09 selectively induced ATLL cell death with IC50 values as low as approximately 10 ng/mL, and no signs of adverse effects were observed when JST-TFR09 was applied to the ATLL xenograft mouse model. It is also important to note that in the safety testing of JST-TFR09 in the cynomolgus monkey, we observed no adverse events except for a mild anemia in a few cases (data not shown). Thus, JST-TFR09 may become a promising therapeutic agent for ATLL with minimal adverse treatment effects. In addition to the direct cytotoxic activity, JST-TFR09 showed comparable ADCC to mogamulizumab. Therefore, the anti-TFR1 JST-TFR09 antibody can be more effective for patients with aggressive ATLL as well as indolent ATLL. Clinical trials using JST-TFR09 may offer important advances in the treatment of ATLL.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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References

[1] K. Ishitsuka, K. Tamura, Human T-cell leukaemia virus type I and adult T-cell leukaemia-lymphoma. Lancet Oncol. 15 (2014) e517-526.

[2] A. Utsunomiya, I. Choi, D. Chihara, et al., Recent advances in the treatment of adult T-cell leukemia-lymphomas. Cancer Sci. 106 (2015) 344-351.

[3] Y. Takasaki, M. Iwanaga, Y. Imaizumi, et al., Long-term study of indolent adult T-cell leukemia-lymphoma. Blood 115 (2010) 4337-4343.

[4] H. Sasaki, I. Nishikata, T. Shiraga, et al., Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. Blood 105 (2005) 1204-1213.

[5] I.C. Moura, Y. Lepelletier, B. Arnulf, et al., A neutralizing monoclonal antibody (mAb A24) directed against the transferrin receptor induces apoptosis of tumor T lymphocytes from ATL patients. Blood 103 (2004) 1838-1845.

[6] H.O. Habashy, D.G. Powe, C.M. Staka et al., Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen. Breast Cancer Res Treat. 119 (2010) 283-293.

[7] E. Ryschich, G. Huszty, H.P. Knaebel, et al., Transferrin receptor is a marker of malignant phenotype in human pancreatic cancer and in neuroendocrine carcinoma of the pancreas. Eur J Cancer. 40 (2004) 1418-1422.

[8] A. Płoszyńska, K. Ruckemann-Dziurdzińska, A. Jóźwik, et al., Cytometric evaluation of transferrin receptor 1 (CD71) in childhood acute lymphoblastic leukemia. Folia Histochem Cytobiol. 50 (2012) 304-311.

[9] J.M. Wu, M.J. Borowitz, E.G. Weir. The usefulness of CD71 expression by flow cytometry for differentiating indolent from aggressive CD10+ B-cell lymphomas. Am J Clin Pathol. 126 (2006) 39-46.

[10] T.R. Daniels, T. Delgado, J.A. Rodriguez, et al., The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol. 121 (2006) 144-158.

[11] D. Brooks, C. Taylor, B. Dos Santos, et al., Phase Ia trial of murine immunoglobulin A antitransferrin receptor antibody 42/6. Clin Cancer Res. 1 (1995) 1259-1265.

[12] R. Crépin, A.L. Goenaga, B. Jullienne, et al., Development of human single-chain antibodies to the transferrin receptor that effectively antagonize the growth of leukemias and lymphomas. Cancer Res. 70 (2010) 5497-5506.

[13] G. Kurosawa, Y. Akahori, M. Morita, et al., Comprehensive screening for antigens overexpressed on carcinomas via isolation of human mAbs that may be therapeutic. Proc Natl Acad Sci U S A. 105 (2008) 7287-7292.

[14] Y. Akahori, G. Kurosawa, M. Sumitomo, et al., Isolation of antigen/antibody complexes through organic solvent (ICOS) method. Biochem Biophys Res Commun. 378 (2009) 832-835.

[15] K. Nagai, S. Nakahata, S. Shimosaki, et al., Development of a complete human anti-human transferrin receptor C antibody as a novel marker of oral dysplasia and oral cancer. Cancer Med. 3 (2014) 1085-1099.

[16] Nakahata S, Saito Y, Marutsuka K, et al., Clinical significance of

CADM1/TSLC1/IgSF4 expression in adult T-cell leukemia/lymphoma. Leukemia 2012 26 (2012) 1238-1246.

[17] S.V. Torti, F.M. Torti. Iron and cancer: more ore to be mined. Nat Rev Cancer 13 (2013) 342-355.

[18] R. Niwa, E. Shoji-Hosaka, M. Sakurada, et al., Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. Cancer Res. 64 (2004) 2127-2133.

[19] D.H. Manz, N.L. Blanchette, B.T. Paul, et al., Iron and cancer: recent insights. Ann N Y Acad Sci. 368 (2016) 149-161.

[20] D. Galaris, V. Skiada, A. Barbouti. Redox signaling and cancer: the role of "labile" iron. Cancer Lett. 266 (2008) 21-29.

[21] T. Moroishi, T. Yamauchi, M. Nishiyama, et al. HERC2 targets the iron regulator FBXL5 for degradation and modulates iron metabolism. J Biol Chem. 289 (2014) 16430-16441.

[22] T.R. Daniels, E. Bernabeu, J.A. Rodríguez, et al., The transferrin receptor and the targeted delivery of therapeutic agents against cancer. Biochim Biophys Acta 1820 (2012) 291-317.

[23] S. White, R. Taetle, P.A. Seligman, et al., Combinations of anti-transferrin receptor monoclonal antibodies inhibit human tumor cell growth in vitro and in vivo: evidence for synergistic antiproliferative effects. Cancer Res. 50 (1990) 6295-6301.

[24] R. Taetle, Dos B. Santos, Y. Ohsugi, et al. Effects of combined antigrowth factor receptor treatment on in vitro growth of multiple myeloma. J Natl Cancer Inst. 86 (1994) 450-455.

Figure Legends

Fig. 1. Overexpression of TFR1 in ATLL.

(**A**) Quantitative RT-PCR analysis for TFR1 in T-ALL and ATLL-related cell lines (left) and primary ATLL cells from acute-type ATLL patients (right). $CD4^+$ T-lymphocytes from healthy volunteers ($CD4^+$ T) were used as controls. The relative TFR1 mRNA expression levels of each sample were normalized by the expression level of β-actin mRNA, and that of the $CD4^+$ T-lymphocytes from healthy volunteer #1 was set to a value of 1. Values are means ± SD; * $P < 0.05$ compared to control group.

(**B**) Immunoblot analysis for TFR1, IRP2, and FBXL5 in T-ALL and ATLL-related cell lines (left) and primary ATLL cells from acute-type ATLL patients (right). $CD4^+$ T-lymphocytes from healthy volunteers ($CD4^+$ T) were used as controls. β-actin was included as a loading control.

(**C**) Flow cytometry histogram plots of surface TFR1 on ATLL-related cell lines and primary leukemic cells from acute-type ATLL patients (Pt #1 and #2). Histograms show fluorescence intensity versus cell count and represent unstained control (blue) and cells stained with JST-TFR09 (red) with percent numbers of cells positive and negative.

Fig. 2. Direct cytotoxic activity of the anti-TFR1 mAb JST-TFR09 against ATLL cells.

(**A**) Survival curve for resting or PHA-activated $CD4^+$ T-cells treated with increasing doses of JST-TFR09 mAb or human immunoglobulin (hIgG) as a control for 96 hours

(left). Values are means ± SD; * $P < 0.05$ compared to control. Cell surface expression of TFR1 and the IL-2Rα chain (CD25) as an indicator of T-cell activation in the untreated samples was determined by flow cytometry (right). Histograms show fluorescence intensity versus cell count and represent unstained control (blue) and cells stained with JST-TFR09 or anti-CD25 antibody (red) with percent numbers of cells positive and negative.

(**B**) Survival curve for ATLL-related cell lines after treatment with the indicated doses of JST-TFR09 mAb for 96 hours. Values are means ± SD; *, #, ‡, $, ¥, or + $P < 0.05$, MT-2, HUT102, SU9T01, ED, S1T, KK1 compared to control, respectively.

(**C**) Survival curve for primary ATLL cells from two acute-type ATLL patients after treatment with the indicated doses of JST-TFR09 mAb or hIgG for 96 hours. Values are means ± SD; * $P < 0.05$ compared to control.

Fig. 3. The anti-TFR1 mAb JST-TFR09 is capable of not only blocking cellular iron uptake but also inducing ADCC against ATLL cells.

(**A**) A competitive binding assay between the TF-TFR1 complex and JST-TFR09 or holo-TF. Relative inhibition of TF-TFR1 binding was determined with increasing amounts of JST-TFR09 mAb or holo-TF, and the background OD450 value was set to 1, representing 100% inhibition of TF-TFR1 binding. Values are means ± SD.

(**B**) Transferrin incorporation assay with the K562 cell line treated with increasing amounts of JST-TFR09 or holo-TF and incubated with fluorescently labeled holo-TF (FL-holo-TF), as determined by flow cytometry measurement of internalization of

FL-holo-TF.

(**C**) Transferrin incorporation assay with the MT-2 cell line pretreated with JST-TFR09 or hIgG and incubated with Alexa Fluor 488-holo-TF for the indicated time. The fluorescence intensity of internalization of Alexa Fluor 488-holo-TF was determined by flow cytometry. Values are means ± SD; * $P < 0.05$ compared to control.

(**D**) ADCC activities were evaluated by measuring the viability of ATLL-related cell lines after incubation with the indicated doses of JST-TFR09 mAb or mogamulizumab and PBMCs from healthy volunteers as effector cells for 8 h. Values are means ± SD; * $P < 0.05$ compared to control (no treatment).

Fig. 4. Effect of the anti-TFR1 mAb JST-TFR09 on tumor growth in mouse xenograft models with ATLL cells.

(**A**) Volume of ATLL cell xenograft tumors in NOG mice treated with 10 mg/kg/day of JST-TFR09, mogamulizumab, or PBS 4 times every 3 days starting 20 days after the ATLL cell transplant. The MT-2 cell line was used for transplantation. The arrow indicates the time of antibody injection. *; $P<0.05$ compared with the controls.

(**B**) Kaplan-Meier survival curves of the MT-2 cell-bearing NOG mice treated with JST-TFR09 or PBS. The arrow indicates the day of injection. *; $P<0.05$; log-rank test; compared with the controls.

(**C**) Volume of CTCL cell xenograft tumors in SCID mice treated with 20 mg/kg/day of JST-TFR09, mogamulizumab, or PBS 5 times every 3 days starting 8 days after the CTCL cell transplant. The arrow indicates the time of antibody injection. *; $P<0.05$

compared with the controls.

Figure

Fig. 1

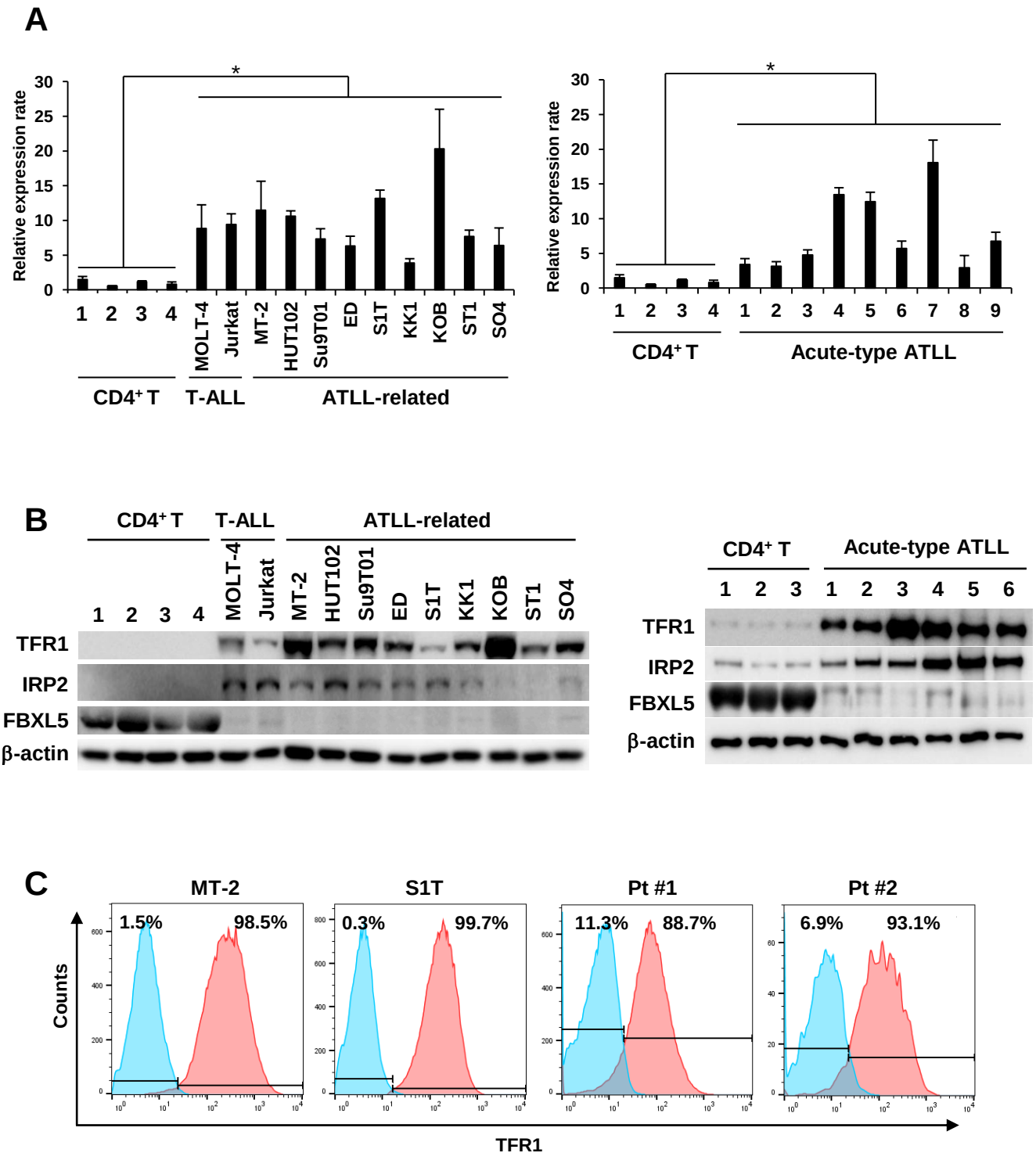

A
Relative expression rate
30
25
20
15
10
5
0
*
1 2 3 4 MOLT-4 Jurkat MT-2 HUT102 Su9T01 ED S1T KK1 KOB ST1 SO4
CD4+ T
T-ALL
ATLL-related
1 2 3 4 1 2 3 4 5 6 7 8 9
CD4+ T
Acute-type ATLL
B
CD4+ T
T-ALL
ATLL-related
1 2 3 4 MOLT-4 Jurkat MT-2 HUT102 Su9T01 ED S1T KK1 KOB ST1 SO4
TFR1
IRP2
FBXL5
β-actin
CD4+ T
Acute-type ATLL
1 2 3 1 2 3 4 5 6
TFR1
IRP2
FBXL5
β-actin
C
MT-2
S1T
Pt #1
Pt #2
1.5%
98.5%
0.3%
99.7%
11.3%
88.7%
6.9%
93.1%
Counts
TFR1

Fig. 2

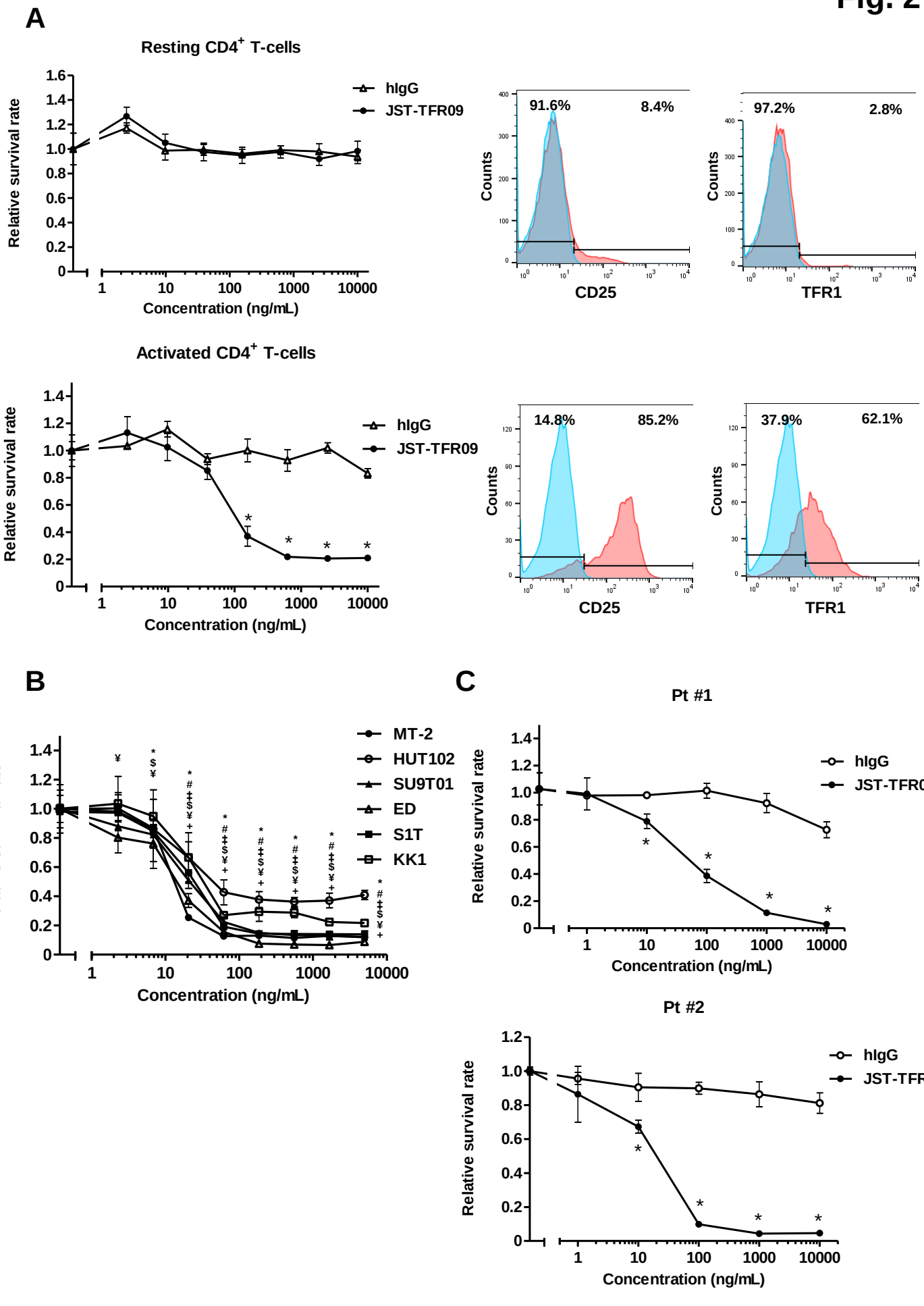

A
Resting CD4+ T-cells
Relative survival rate
Concentration (ng/mL)
hIgG
JST-TFR09
91.6%
8.4%
97.2%
2.8%
Counts
CD25
TFR1
Activated CD4+ T-cells
14.8%
85.2%
37.9%
62.1%
B
MT-2
HUT102
SU9T01
ED
S1T
KK1
C
Pt #1
Pt #2

Fig. 3

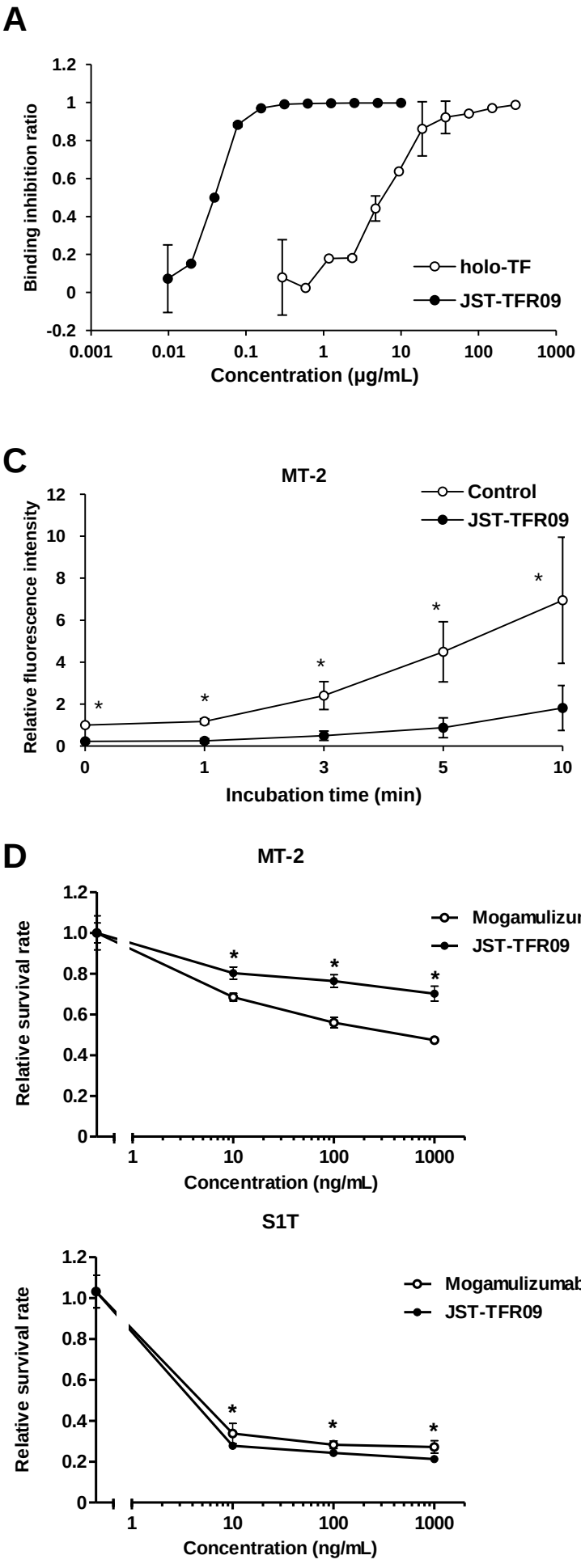
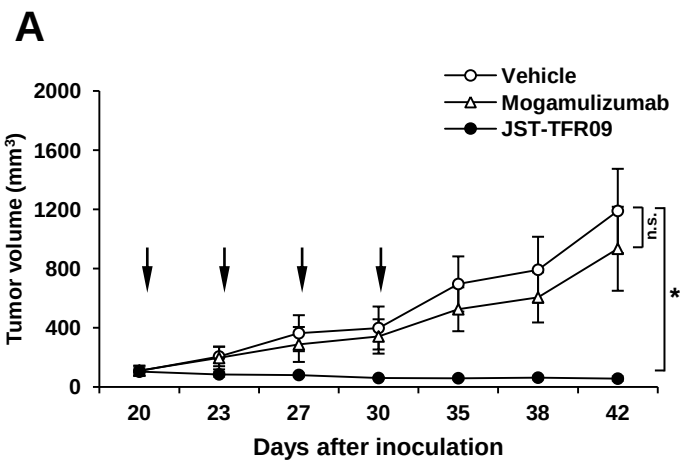
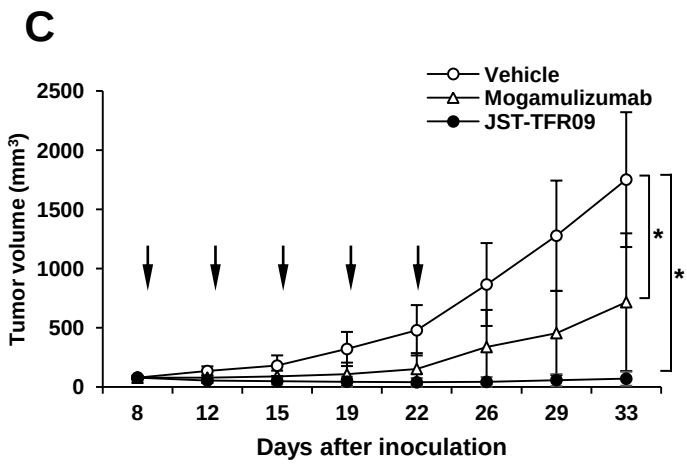
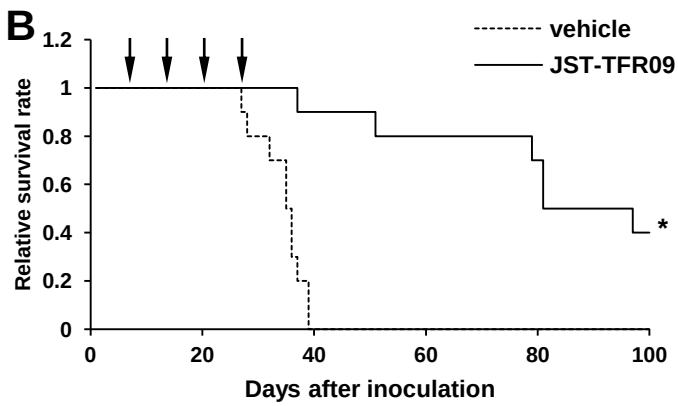

A
Binding inhibition ratio
Concentration (μg/mL)
holo-TF
JST-TFR09
B
K562
FL-holo-TF
Counts
FL-holo-TF with holo-TF (unlabeled)
100000
6250
390
24
1.5
0.0
(ng/mL)
FL-holo-TF with JST-TFR09
C
MT-2
Control
JST-TFR09
Relative fluorescence intensity
Incubation time (min)
D
MT-2
Mogamulizumab
JST-TFR09
Relative survival rate
Concentration (ng/mL)
S1T
Mogamulizumab
JST-TFR09
Relative survival rate
Concentration (ng/mL)

Fig. 4


A
Vehicle
Mogamulizumab
JST-TFR09
Tumor volume (mm3)
2000
1600
1200
800
400
0
n.s.
*
20
23
27
30
35
38
42
Days after inoculation


C
Vehicle
Mogamulizumab
JST-TFR09
Tumor volume (mm3)
2500
2000
1500
1000
500
0
*
*
8
12
15
19
22
26
29
33
Days after inoculation


B
vehicle
JST-TFR09
Relative survival rate
1.2
1
0.8
0.6
0.4
0.2
0
*
0
20
40
60
80
100
Days after inoculation

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***Conflict of Interest**

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